Expression, purification and characterization of a human serine-dependent phospholipase A_2 with high specificity for oxidized phospholipids and platelet activating factor

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Using expressed sequence tag (EST) homology screening, a new human serine dependent phospholipase A_2 (HSD-PLA_2) was identified that has 40% amino acid identity with human low density lipoprotein-associated phospholipase A_2 (LDL-PLA_2). HSD-PLA_2 has very recently been purified and cloned from brain tissue but named PAF-AH II. However, because the homology with LDL-PLA_2 suggested a broader substrate specificity than simply platelet activating factor (PAF), we have further characterized this enzyme using baculovirus-expressed protein. The recombinant enzyme, which was purified 21-fold to homogeneity, had a molecular mass of 44 kDa and possessed a specific activity of 35 μmol min^{-1} mg^{-1} when assayed against PAF. Activity could also be measured using 1-decanoyl-2-(4-nitrophenylglutaryl) phosphate (DNGP) as substrate. Like LDL-PLA_2, HSD-PLA_2 was able to hydrolyse oxidatively modified phosphatidylcholines when supplemented to human LDL prior to copper-stimulated oxidation. A GXSXG motif evident from sequence information and inhibition of its activity by 3,4,dichloroisocoumarin, diisopropyl fluorophosphate (DFP) and diethyl *p*-nitrophenyl phosphate (DENP) confirm that the enzyme is serine dependent. Moreover, sequence comparison indicates the HSD-PLA_2 probable active site triad positions are shared with LDL-PLA_2 and a *C. elegans* homologue, suggesting that these sequences comprise members of a new enzyme family. Although clearly structurally related with similar substrate specificities further work reported here shows HSD-PLA_2 and LDL-PLA_2 to be different with respect to chromosomal localization and tissue distribution.

INTRODUCTION

Several platelet activating factor (PAF) acetyl hydrolase activities have been reported in various mammalian tissues [1,2]. Until recently only two proteins had been unequivocally identified which hydrolyse PAF, human plasma LDL-PLA_2 [3,4] and a bovine brain PAF acetylhydrolase [5]. Despite both enzymes being serine dependent enzymes, with the ability to hydrolyse PAF, they are remarkably different. The plasma enzyme is a single polypeptide containing a lipase consensus motif, GXSXG [6] and is expressed in inflammatory cells, in particular macrophages, or in tissues having a high inflammatory cell content. In addition to having PAF acetylhydrolase activity, the plasma enzyme also possesses high phospholipase activity towards phosphatidylcholines with either oxidized or short chain fatty acids in the *sn*-2 position [3,7]. The bovine brain enzyme is an α, β, γ heterotrimeric complex with catalytic serine residues in both the β and γ subunits [8].

Two other PAF acetylhydrolase activities which have been described include an erythrocyte enzyme which is reported to be a 25 kDa homodimeric protein [9] but has not been ascribed to a defined protein sequence, and an intracellular bovine enzyme found in brain, kidney and liver [2]. This bovine enzyme and its human homologue have very recently been reported as 44 kDa polypeptides (platelet-activating factor acetylhydrolase II; [10]). Both plasma and intracellular PAF-acetylhydrolases have recently been reviewed [11].

In the present study we report the stable expression, purification and further characterization of this newly described human enzyme. We have demonstrated that this calcium-independent enzyme has a broad spectrum of substrates, in line with phospholipase activity. In contrast to LDL-PLA_2, HSD-PLA_2 appears to be an intracellular enzyme with broad tissue distribution. The data presented here clearly demonstrate that LDL-PLA_2 and HSD-PLA_2 are related proteins with similar substrate specificities but different chromosomal localization and tissue distribution.

EXPERIMENTAL

Materials

All cell culture medium was supplied by Life Technologies. Buffer A contained 5 mM CHAPS, 1 mM EDTA, 1 mM DTT, 20% v/v glycerol. Cell lysis buffer contained 50 mM Tris in buffer A, pH 8.5, plus 0.01 mg ml^{-1} protease inhibitors, pepstatin

Abbreviations used: DENP, diethyl *p*-nitrophenyl phosphate; DFP, diisopropyl fluorophosphate; DNGP, 1-decanoyl-2-(4-nitrophenylglutaryl) phosphate; DTT, dithiothreitol; EST, expressed sequence tag; HSD-PLA_2 human serine dependent phospholipase A_2; LDL, low density lipoprotein; LDL-PLA_2, lipoprotein-associated phospholipase A_2; PAF, platelet activating factor; PAF-AH, PAF acetylhydrolase; PtdCho, phosphatidylcholine; RH, radiation hybrid; Sf9, *Spodoptera frugiperda* 9; STS, sequence tag site.

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A, antipain, aprotinin and leupeptin and 0.05 mg ml^{-1} benzamidine. All buffer reagents and *p*-nitro phenyl acetate were obtained from Sigma. DNase was obtained from Boehringer Mannheim. Q Sepharose (high performance), blue Sepharose 6FF, and resource Q were obtained from Pharmacia. Macro-prep. ceramic hydroxyapatite was obtained from Bio-rad. The micro BCA assay was manufactured by Pierce & Warriner. ^{3}[H]PAF was manufactured in-house.

Cloning and baculovirus expression of HSD-PLA_2

The initial expressed sequence tag (EST) clones used in the study were prepared by scientists at Human Genome Sciences using established EST methods [12,13]. Accumulated EST data facilitated the assembly of a large multiple cDNA library contig and several full length cDNAs were subsequently identified. Double stranded DNA sequencing via the dideoxy chain termination method (Sequenase; Amersham) and general molecular cloning techniques were carried out according to standard protocols [14–16]. Generation of recombinant baculovirus to infect the cultured insect cell line *Spodoptera frugiperda* 9 (SF-9) and so express HSD-PLA_2 was carried out according to standard procedures [17]. Briefly, HSD-PLA_2 was isolated from pBluescript SK via *Eco*RI/*Xho*I restriction endonuclease digestion, the DNA 5′ overhangs were filled using the Klenow fragment of DNA polymerase I and inserted into the *Sma*I site of the pBacPAK 9 transfer vector (Clontech). This new plasmid was co-transfected with *Bsu*36I digested BacPAK 6 viral DNA (Clontech) via lipofection (Cellfectin; Life Technologies) into SF-9 cells. Two days post transfection, recombinant virus released into the culture medium was used to establish a plaque assay from which single plaques were picked for virus amplification prior to analysis for enzymatic activity. Virus titre and cell harvest time post infection were optimized for peak protein production.

Radiation hybrid (RH) mapping

Synthetic oligonucleotides (Cruachem, U.K.) were obtained to amplify products from the HSD-PLA_2 3′ untranslated region and LDL-PLA_2 coding regions. HSD-PLA_2 primers used were (5′ → 3′) TCACACTGCTGCTTGGATAA and TTTTTCACGGCTCACACC. LDL-PLA_2 primers used were (5′ → 3′) GATGTGGTATTGCCCTGGATGCATGG and CTCCTTCAATCAAGCAGTCCCACTG. The PCR reactions generated fragments of the expected sizes, 340 and 450 bp respectively.

Oligonucleotide pairs were used for PCR of HSD-PLA_2 and LDL-PLA_2 against the 96 samples of the GeneBridge 4 radiation hybrid panel (Research Genetics; [18]), twice using the same 2 step PCR conditions, 94 °C for 40 s, 60 °C for 30 s, 35 cycles in 20 μl reactions containing 1 U KlenTaq1 (GenPak Ltd.), 50 mM Tris/HCl, pH 9.1, 16 mM ammonium sulphate, 3.5 mM $MgCl_2$, 150 μg ml^{-1} bovine serum albumin, plus 25 ng of GeneBridge 4 RH panel DNA. The panel includes three controls: donor DNA, host DNA and water. Samples were electophoresed on 3% agarose gels and each RH sample was scored for presence (1) or absence (0) of a band of the correct size. In the case of a discrepancy for any sample between the two RH PCR runs, that sample was scored as ambiguous (2). Cytogenetic locations were inferred from these results using the markers shown to neighbour the genes via the Genome Directory [19] and the Genome Database [20] (http://gdbwww.gdb.org/gdb/docs/gdbhome.html). Chromosomal assignments were confirmed by PCR on the BIOS panel (BIOS, U.S.A.) using the same oligonucleotide pairs and conditions as for the RH mapping.

Sequence searching and analysis

Comparative analysis was done with the Genetics Computer Group (GCG) program suite version 8.0 [21]. The major public protein and nucleotide databases were searched using BLAST [22] on the National Centre for Biotechnology Information databases server (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). The Human Genome Sciences EST collection was searched by BLAST on an in-house server [23].

Assays

HSD-PLA_2 activity was measured according to the method described for LDL-PLA_2 [3] using either 1-decanoyl-2-(4-nitrophenylglutaryl) phosphate (DNGP) or [^{3}H]PAF as a substrate. PAF was used in the range 2–400 μM, whilst DNGP was used in the range 2–300 μM. All assays were performed at 37 °C in 50 mM Hepes, 150 mM NaCl, 5 mM CHAPS, 1 mM EDTA and 20% v/v glycerol pH 7.4 unless stated otherwise. DNGP was prepared as a 10 mM stock solution in methanol and diluted into buffer as required. The absorbance increase on addition of HSD-PLA_2 was followed at 400 nm, using either a diode array spectrophotometer (Hewlett–Packard) or a 96-well plate reader (Molecular Devices, Tmax) running in kinetic mode. Protein content of purification fractions was measured using the micro BCA assay.

Purification of baculovirus expressed enzyme

Sf9 cells expressing HSD-PLA_2 were lysed by freeze/thawing in liquid nitrogen following the addition of 40 ml l^{-1} cell lysis buffer. After cell lysis, DNase was added to reduce the viscosity of the sample and the lysate was spun at 10000 *g* for 30 min to pellet the cell debris. The resulting supernatant was loaded onto a Q Sepharose column (2.6 × 20 cm). The column was washed with 50 mM Tris in buffer A, pH 8.5, and eluted with a gradient of 0–100% buffer A plus 1.0 M NaCl. Enzyme activity eluted at 200 mM NaCl. Active fractions were pooled and the pH of the sample was lowered to 6.0 using 1 M Mes in buffer A ready to load onto a blue Sepharose 6FF column (5 × 15 cm). Prior to loading, the sample was diluted to 1 litre with 50 mM Mes in buffer A, pH 6.0. The column was washed with 50 mM Mops, in buffer A, plus 0.5 M NaCl pH 7.0, before elution with 50 mM Tris in buffer A, plus 1.0 M NaCl, pH 8.5. Active fractions were pooled and concentrated by ultrafiltration (Amicon YM30) before dialysis overnight against 10 mM Tris, 5 mM CHAPS, 1 mM DTT, 20% v/v glycerol, pH 7.4. Following dialysis any precipitates were removed by centrifugation and the HSD-PLA_2 was then loaded onto a hydroxyapatite column (1.6 × 10 cm). The column was washed with 10 mM Tris, 5 mM CHAPS, 1 mM DTT, 20% v/v glycerol, pH 7.4 and eluted with a gradient of 0–100% 150 mM KH_2PO_4. The activity eluted at 50 mM KH_2PO_4. Active fractions were pooled, concentrated and then diluted with 50 mM Tris, in buffer A, pH 8.5, before loading onto a 6 ml Resource Q column. The column was washed with loading buffer. Active protein elutes in the void volume which was concentrated and stored at 4 °C.

Purity analysis and structural characterization

Fractions from each of the major purification steps were analysed by SDS/PAGE as described [3]. The final preparation was also analysed by reverse-phase HPLC using a 0.75 mm × 85 mm plastic microbore column packed with Poros (Perceptive Biosystems) as described [24]. Solvent A contained 0.08% trifluoroacetic acid and solvent B contained 0.08% trifluoroacetic

acid and 80% acetonitrile. A sample of 50 μl was injected and eluted with a gradient of 30% to 80% solvent B in 5 min.

N-terminal sequencing was done as described for LDL-PLA_2 [3]. Proteins from the SDS/PAGE analysis of purified enzyme were blotted from the gel onto PVDF membrane (Bio-Rad). Bands stained with Coomassie Blue were excised and placed in a blot cartridge of an Applied Biosystems/Perkin–Elmer 477a Protein Sequencer. Mass determination of the intact protein was carried out by liquid chromatography MS on a Finnigan TSQ 700 instrument equipped with an electrospray source.

Hydrolysis of oxidized phospholipids

LDL (0.7 mg ml^{-1} protein) was isolated and endogenous LDL-PLA_2 inhibited by treatment with 2 mM diethyl *p*-nitrophenyl phosphate (DENP) for 60 min as reported previously [3]. After DENP treatment, LDL was gel filtered to remove EDTA and unreacted DENP on a Superdex 200 (prep. grade) column (1.6 × 35 cm) pre-equilibrated with phosphate buffered saline as running buffer. The LDL was divided into two fractions. After the addition of 5 μM Cu^{2+} to one fraction, oxidation was allowed to proceed at 37 °C for 3 h. The extent of conjugated diene formation was monitored at 234 nm. Significant oxidation occurred only in the Cu^{2+} treated LDL. Oxidation was stopped by the addition of 1 mM EDTA and 20 μM 3,5-di-*t*-butyl-4-hydroxytoluene. The Cu^{2+} oxidized LDL was divided into five aliquots and the following additions made: no addition, 0.1 μg ml^{-1} purified LDL-PLA_2 and 0.1,1 and 10 μg ml^{-1} purified HSD-PLA_2. After incubation at 37 °C for a further 60 min samples were extracted and the lyso-phosphatidylcholine (lyso-PtdCho) content determined as described previously [3]. PAF acetyl hydrolase activity was measured using 100 μM ^{3}[H]PAF.

Antibody generation, immunoadsorption and tissue localization studies

Polyclonal antibodies against human HSD-PLA_2 were prepared in rabbits (2.5 kg) by using standard protocols. Briefly, a 1:1 emulsion of purified HSD-PLA_2 in saline and Freund's complete adjuvant was injected subcutaneously into four sites. Identical booster injections of antigen (50–100 μg) with incomplete adjuvant were given 4 weeks later. High antibody titre was observed 6–7 weeks after the initial immunization.

Cell extracts were prepared by firstly washing cells in PBS then resuspending the cell pellets in 1.5 ml homogenization buffer (50 mM Tris, 10 mM CHAPS, 2 mM EDTA, 2 mM EGTA, pH 8.0) containing a protease inhibitor cocktail, comprising pepstatin A, antipain, protein A and leupeptin using a 1/1000 dilution of a 1 mg ml^{-1} stock solution prepared in ethanol. Cells were homogenized using a cycle of freeze/thawing (× 3) in liquid nitrogen. Cellular extracts to be used in the immunoadsorption assays were prepared by centrifuging the homogenates for 20 min at 90000 *g*, 4 °C, in a Beckman TL100 ultracentrifuge. Immunoadsorption analysis using solid-phase reagents (protein-A agarose based) coupled to rabbit serum raised against recombinant human HSD-PLA_2 was carried out as detailed previously [25].

Tissue localization was carried out by PCR, using specific primers designed from the HSD-PLA_2 coding sequence, screening a panel of cDNAs derived from eight different cDNA libraries (QuikScreen-Clontech). Primers used were (5′ → 3′) CACGTGGCTTTGTGGTTGCTGTGC and GTTATCCAAGCAGCAGTGTGA, respectively. Amplification was achieved with 30 successive rounds of 94 °C for 1 min followed by 60 °C for 1 min and 72 °C for 1 min using 1.25 mM $MgCl_2$, 500 nM of each primer, 200 nM dNTPs and 0.5 U of taq DNA polymerase. Aliquots of cDNA from the Quikscreen panel were used according to the manufacturer's guidelines. Specific brain region localization was achieved using RT-PCR from mRNA isolated from human tissues using a similar amplification programme.

RESULTS

Sequence characterization of human HSD-PLA_2

A comprehensive database search was performed with the HSD-PLA_2 sequence. Including the main non-redundant databases and monthly updates, a total of 275138 protein sequences and 337339 nucleotide sequences were searched, in addition to dbEST, with 1147640 sequences. All 12 protein database hits with probabilities of less than 10^{-5} are shown in Table 1. This established that HSD-PLA_2, cloned independently in this work, was identical to the protein recently reported as PAF-acetylhydrolase II. The 30–40% overall sequence similarity with all six species orthologues of LDL-PLA_2 is reflected in sequence probability matches of below 10^{-100} and the conservation of the active site triad positions between all the top eight database sequences. Interestingly *C. elegans* contains an orthologue of the same enzyme family. The DNA comparisons not only showed significant similarity between all eight vertebrate sequences but also with the *C. elegans* enzyme. Inspection of the protein alignments for the last three entries in Table 1 show these to be restricted only to the vicinity of the active site serine and N-terminal section common to all three sequences. Although the amino acid alignments show only 25–35% identity over 30–50 residues, the BLAST probabilities of less than 10^{-5} are significant and may reflect a common α/β hydrolase fold architecture for these distant prokaryotic lipase homologues. A reading-frame search of the EST database showed two human entries with sequence identity to HSD-PLA_2, gb|AA298364 from pineal gland and gb|AA297363 from foetal heart. All other human and mouse dbEST matches were attributable to LDL-PLA_2 sequences.

Radiation hybrid mapping

The MIT markers that show tightest linkage to HSD-PLA_2 are the STS markers WI-9053, and RP_L11_1. The closest markers useable for cytogenetic assignment are D1S436 (< 2 cM distal to HSD-PLA_2) and D1S195 (< 4 cM proximal to HSD-PLA_2). The inferred cytogenetic location between these markers is 1p34.3.

Table 1 Protein database search results for HSD-PLA_2

Sequence redundancy has been removed and the names of some entries expanded for clarity. The primary GenBank accession number is given in each case.

Accession no.	Description	Probability
D67845	PAF-acetylhydrolase II, human	2.0×10^{-289}
D87559	PAF-acetylhydrolase II, bovine	2.2×10^{-256}
U34278	PAF-acetylhydrolase, chicken	6.9×10^{-132}
U34246	PAF-acetylhydrolase, dog	8.3×10^{-116}
D67037	PAF-acetylhydrolase, guinea pig	2.4×10^{-114}
U24577	PAF-AH/LDL-PLA_2, human	2.4×10^{-114}
U34247	PAF-acetylhydrolase, bovine	6.1×10^{-114}
U34277	PAF-acetylhydrolase, *mouse*	1.9×10^{-111}
U64598	*C. elegans*, similar to PAF-AH	6.7×10^{-56}
M86351	28 K lipase precursor – *Streptomyces* sp.	5.7×10^{-6}
U14003	Hypothetical protein from *E. coli*	7.1×10^{-5}
D90911	Hypothetical protein from *Synechocystis*	8.1×10^{-5}

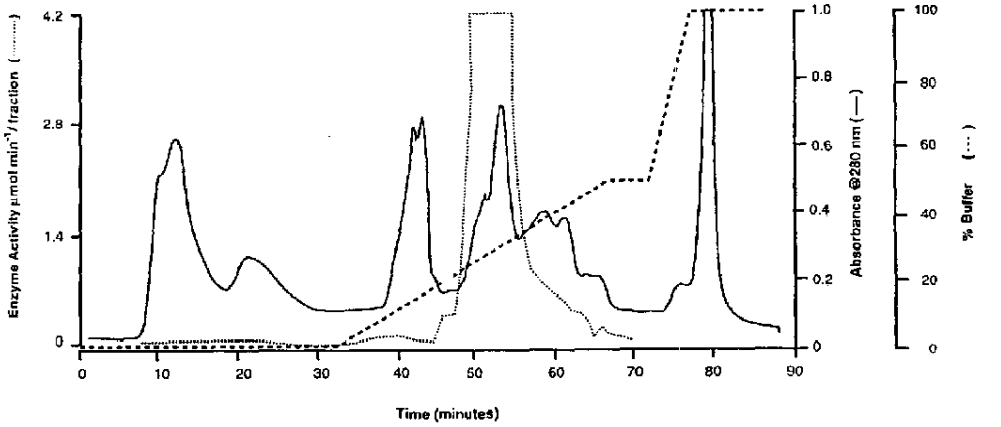

Figure 1 Purification of crude HSD-PLA$_2$ on a high performance Q Sepharose column

The enzyme activity of fractions was followed using 50 μM DNPG, as described in the Experimental section.

These data were confirmed by FISH analysis (Genome Sciences; data not shown). The PAF receptor RH data are available on the MIT WWW server. When the HSD-PLA$_2$ and PAF receptor Rh data are entered together, the RH mapping server places them 0.7 cRays apart, the PAF receptor being the more proximal. The LDL-PLA$_2$ gene is flanked by the STS markers D6S459 and D6S452, an interval of approx. 5 cRays (1–2 cM). The inferred cytogenetic location of these STS markers is 6p21. None of the ESTs known to map to this region of chromosome 6 (26) shows any similarity to LDL-PLA$_2$ or HSD-PLA$_2$. A less precise search using OMIM (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim) also revealed no presence of lipase enzymes at 6p21 or 1p34.

Baculovirus expression

In order to examine further the properties of this gene, we expressed the cDNA in a baculovirus system which resulted in high levels of active HSD-PLA$_2$. Optimum expression was observed 48 h post infection and yielded 60 mg l^{-1}. Immunoadsorption studies using a polyclonal antibody raised against the purified material isolated greater than 95% of the measurable activity present in the insect cell lysate, whilst monoclonal and polyclonal antibodies raised against LDL-PLA$_2$ failed to immunoprecipitate the recombinant HSD-PLA$_2$ (data not shown).

Purification

The very high expression levels obtained in the baculovirus system allowed us to devise a relatively simple four-step, high yield, purification of recombinant HSD-PLA$_2$. Only one significant enzyme activity, as judged by both PAF and DNGP hydrolysis, was present in crude baculovirus infected Sf9 cells. Glycerol and DTT were found to be essential to maintaining good enzyme activity whilst CHAPS significantly improved the yield of all chromatography steps, suggesting that the protein is somewhat hydrophobic and prone to sticking to columns or chromatography matrices. The inclusion of EDTA was also found to be beneficial, particularly at the earlier stages where unwanted proteolysis is likely to be a problem. Although HSD-PLA$_2$ is found to bind to a Q Sepharose column in its crude form

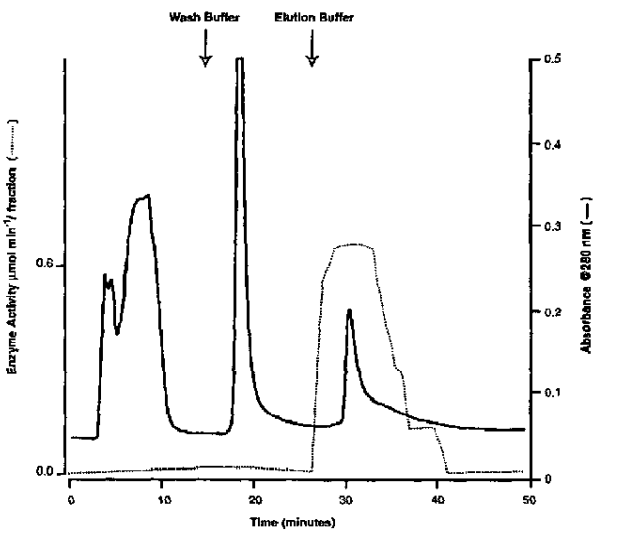

Figure 2 Purification of HSD-PLA$_2$ on a blue Sepharose 6 FF column

The enzyme activity of fractions was followed using 50 μM DNPG, as described in the Experimental section.

(Figure 1), it is interesting to note that in the final purification step it is not possible to bind HSD-PLA$_2$ to a putatively similar matrix (Resource Q) under identical conditions. It is not clear whether this is due to a difference in ion exchange matrices or whether HSD-PLA$_2$ binds to other proteins in the crude Sf9 homogenate. As with the related protein, LDL-PLA$_2$, the use of Blue Sepharose 6FF was found to be of value. However, HSD-PLA$_2$ elutes at a much lower salt concentration than LDL-PLA$_2$ which reduces the purification available from this step (Figure 2). It is believed that the presence of non-proteinaceous UV absorbing material could give rise to the elution profiles shown, compared with the low purification factor achieved from the first

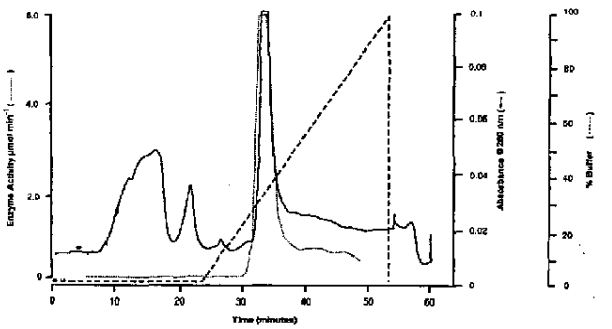

Figure 3 Purification of HSD-PLA_2 on a hydroxyapatite column

The enzyme activity of fractions was followed using 50 μM DNPG, as described in the Experimental section.

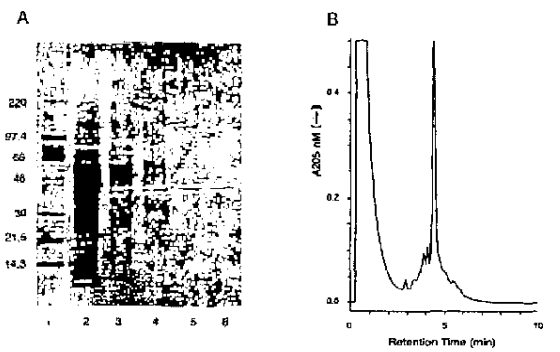

Figure 4 Purity analysis of recombinant HSD-PLA_2

The purification of HSD-PLA_2 was followed by SDS/PAGE with Coomassie blue staining (**A**) as described in the Experimental section. Lane 1, molecular weight markers (kDa); Lane 2, crude supernatant; Lane 3, post Q column; Lane 4, post Blue Sepharose column; Lane 5, post hydroxyapatite column; Lane 6, post resource Q column. The purity of the material following the post resource Q column was analysed by reverse phase HPLC (**B**) as described in the Experimental section.

Table 2 Typical purification of recombinant HSD-PLA_2

The data are taken from a single enzyme preparation starting with 5 litres of Sf9 cells.

Step	Activity (AU min^{-1})	Protein (mg)	Specific activity (AU min^{-1} mg^{-1})	Yield (%)	Purification factor
Crude supernatant	3360	2840	1.2		
Q Sepharose HP	3400	2080	1.6	100	1.4
Blue Sepharose-6-Fast Flow	2600	1070	2.5	78	2.1
Hydroxyapatite	1800	160	11	54	9.5
Resource Q	1500	60	25	45	21

two columns. Finally, inclusion of a hydroxyapatite column (Figure 3) followed by a final pass over a Resource Q column removes the remaining impurities and gives HSD-PLA_2 which shows only a single band by SDS/PAGE (Figure 4a, lane 6).

Table 3 Class specific inhibitor results

Inhibitors were pre-incubated in assay buffer with 0.5 nM enzyme for 10 min at 37 °C. Following the addition of 0.02 mM DNGP, the assay was run for a further 20 min at 405 nM and 37 °C. Inhibition was calculated as a percentage of the activity obtained after 0.5 nM enzyme was pre-incubated in assay buffer in the absence of inhibitors and treated as described above.

Inhibitor (1 mM)	Inhibition (%)
EDTA	0
DFP	20
DENP	36
3,4-Dichloroisocoumarin	100

Purity of the enzyme was confirmed by reverse-phase HPLC (Figure 4b). The purification schedule is summarized in Table 2.

The predicted molecular weight of 44053 Da is in good agreement with the SDS/PAGE results. N-terminal sequencing confirmed M-G-V-N-Q for the intact protein, indicating the absence of any N-terminal peptide processing. However mass spectrometric measurements gave a mass of 44162 Da, suggesting the post-translated addition of 127 Da. The nature and origin of this possible modification is not yet clear. Polyclonal antibodies raised against the purified HSD-PLA_2 recognised greater than 95% of the measurable activity present in the baculovirus lysate and were found not to react with LDL-PLA_2.

Activity assays and inhibitor sensitivity

HSD-PLA_2, like LDL-PLA_2 but unlike classical phospholipases [27,28], shows normal Michaelis–Menten kinetics with both PAF and DNGP as substrates (data not shown). From this result it would appear that HSD-PLA_2 is similar to LDL-PLA_2 in that interfacial activation is not required. The K_m for PAF and DNGP is 42 μM and 10 μM, respectively whilst V_{max} is 35 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 1.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. Thus, although DNGP provides a very straightforward assay for following HSD-PLA_2 activity, it is a markedly slower substrate to turn over than PAF. In addition the enzyme does not turn over *p*-nitrophenyl acetate.

In keeping with the serine dependent lipase motif found in the predicted protein sequence and the homology with LDL-PLA_2, HSD-PLA_2 is found to be sensitive to serine hydrolase specific inhibitors and insensitive to Ca^{2+} and EDTA. These data are summarized in Table 3.

Hydrolysis of oxidized phospholipids

As LDL-PLA_2 has been shown to hydrolyse short chain or oxidized phospholipids almost as efficiently as it hydrolyses PAF, it was of interest to investigate the ability of HSD-PLA_2 to hydrolyse oxidized phospholipids. In order to provide a wide range of possible oxidized phospholipid substrates for HSD-PLA_2, oxidized LDL was used as a potential substrate source rather than any one specific phosphatidylcholine. After oxidation of LDL, which had previously had all LDL-PLA_2 activity removed by DENP treatment [3], various quantities of HSD-PLA_2 were added along with a comparator concentration of pure recombinant LDL-PLA_2. Both PAF acetylhydrolase activity and lyso-PtdCho production were measured. The data are shown in Table 4. From these data it is clear that an addition of 1 $\mu g\ ml^{-1}$ of purified HSD-PLA_2 is equivalent to 0.1 $\mu g\ ml^{-1}$ LDL-PLA_2 in terms of hydrolysing oxidized phosphatidylcholines in LDL. In

Table 4 Lyso-PtdCho production by HSD-PLA_2 and LDL-PLA_2 supplemented, Cu^{2+} oxidized LDL

Freshly prepared human LDL was incubated with either DENP, or vehicle, to abolish endogenous LDL-PLA_2 activity. HSD-PLA_2 and LDL-PLA_2 samples were then added to the DENP-treated LDL and following identical incubation subjected to copper-mediated oxidation as described in Materials and methods. Values represent mean ± S.D. for triplicate incubations from a representative experiment. *$P < 0.01$ when comparing control vs. copper-treated samples.

Supplement	Lyso-PtdCho (μg mg^{-1})	
	No Cu^{2+}	5 μM Cu^{2+}
None	4.82 ± 2.43	7.53 ± 1.65
0.1 μg ml^{-1} HSD-PLA_2	4.31 ± 0.72	6.84 ± 1.11
1.0 μg ml^{-1} HSD-PLA_2	4.64 ± 1.91	16.88 ± 2.11*
10 μg ml^{-1} HSD-PLA_2	4.00 ± 1.47	27.53 ± 2.90*
0.1 μg ml^{-1} LDL-PLA_2	4.18 ± 0.85	18.93 ± 2.52*
No DENP pretreatment, no supplementation	4.95 ± 3.05	22.13 ± 2.83*

Table 5 Immunoadsorption of HSD-PLA_2 in the cytosol of six different human cell types

Cell extracts were prepared, incubated with or without the indicated solid-phase antibody reagents, and assayed against 100 μM PAF as described in the Experimental section. Data are from a single experiment, where the growth of all cell lines was specifically coordinated for this experiment

Cell type	PAF-acetyl hydrolase activity, (pmol · min^{-1} · mg^{-1})		
	Control	+ Preimmune serum	+ Antiserum
Raji	43	38	14
HEK 293	95	86	67
THP-1	65	58	31
T-lymphocytes	76	65	18
Neutrophils	14	13	3
Platelets	24	20	7

the absence of Cu^{2+}-catalysed phospholipid oxidation, HSD-PLA_2, like LDL-PLA_2 [7], does not produce any lyso-PtdCho suggesting that HSD-PLA_2 is unable to hydrolyse PtdCho with long chain fatty acids at the *sn*2 position. This approach to measuring the extent of oxidized PtdCho turnover does not allow any comparison of the rates of turnover.

Immunoadsorption from extracts of various cell types and tissue localization

The immunoadsorption data from the cytosolic fraction of six different cell types are shown in Table 5. It is clear from these data that both T lymphocytes and the immortalized B cell line, Raji cells, contain PAF acetyl hydrolase activity that is mainly attributable to HSD-PLA_2. In contrast the monocyte derived cell line (THP-1 cells) contains a similar amount of PAF acetyl hydrolase activity but at least 50% of this is not ascribed to HSD-PLA_2. This is perhaps not surprising as cells of monocytic origin are known to express LDL-PLA_2 as well. Interestingly, the embryonic kidney cell line, HEK 293, also appears to express a significant quantity of non HSD-PLA_2 PAF acetylhydrolase activity. Neutrophils and platelets, on the other hand, both display relatively little PAF acetylhydrolase activity, although the majority of this activity would appear to be HSD-PLA_2. Thus HSD-PLA_2 would appear to be broadly expressed in all six cell lines examined but B and T lymphocytes appear to show selective high HSD-PLA_2 expression.

These data were complemented by PCR tissue localization studies where HSD-PLA_2 mRNA was detected in all major peripheral tissues examined. Further RT-PCR experiments using mRNA from specific brain regions showed HSD-PLA_2 only to be present in frontal cortex and amygdala of the regions examined. Whilst LDL-PLA_2 expression is more restricted in peripheral tissues, principally regions having cells of monocytic origin, it was found to be more widely expressed in brain regions.

DISCUSSION

In the present study we have further characterized an HSD-PLA_2 that has 40% identity with the previously described LDL-PLA_2. Searching the current databases has identified the first human EST entries from this gene and an early metazoan ancestor from *C. elegans*. Expression of the cDNA in a baculovirus system produced an unprocessed 44 kDa polypeptide as shown by SDS/PAGE mass spectrometric analysis. N-terminal sequencing confirms the predicted absence of signal peptide processing.

The interpreted cytogenetic location from the RH mapping of HSD-PLA_2 places this gene at 1p34.3. A search of the mouse and human genomic databases revealed no obvious candidate disease loci for which HSD-PLA_2 could be implicated. However, it is of interest that the PAF receptor has been mapped to within 500 kb of HSD-PLA_2 [29]. Examination of genomic clones from this region of chromosome 1 is required to establish the actual proximity of these genes. None of the recently mapped ESTs [29] in this region shows any similarity to either HSD-PLA_2 or the PAF receptor. LDL-PLA_2, the only other identified human gene with any similarity to HSD-PLA_2, maps to 6p21 [3].

Expressed protein showed enzymatic activity against the same substrates as LDL-PLA_2, DNGP and PAF, although the hydrolysis rate of DNGP was 100-fold less than that seen with LDL-PLA_2, indicating a potential difference in substrate specificities.

Studies with oxidized LDL as a heterogeneous source of oxidized phospholipids clearly demonstrate that HSD-PLA_2 is capable of hydrolysing oxidized but not native phosphatidylcholines. The relative ability of HSD-PLA_2 to use oxidized phospholipids as substrates compared to PAF is very similar to that seen for LDL-PLA_2. Thus, it seems likely that under physiological conditions oxidized phosphatidylcholines could be substrates for HSD-PLA_2.

Our results suggest the tissue distribution of the HSD-PLA_2 mRNA is different from that of LDL-PLA_2. Immunoadsorption experiments show that HSD-PLA_2 constitutes the major PAF acetyl hydrolase activity in the cytosol of both B and T lymphocytes whilst in cells of a monocytic lineage LDL-PLA_2 predominates. Also, although HSD-PLA_2 is found to be present at relatively low levels in neutrophils and platelets it would appear to be the major PAF-acetylhydrolase activity in these cells. In the brain, HSD-PLA_2 mRNA expression appears to be restricted to the amygdala and frontal cortex, whilst LDL-PLA_2 expression was detected in all regions examined. The biological significance of this tissue distribution and the relationship between HSD-PLA_2 and LDL-PLA_2 activities in various cell types requires further elucidation.

Given that HSD-PLA_2 is likely to be an intracellular enzyme it is interesting to speculate on what role such an enzyme would play in either PAF metabolism or the hydrolysis of oxidized

phospholipids. As PAF effects are mediated in an extracellular fashion it is difficult to see what role an intracellular PAF acetylhydrolase would have in protecting against the unknown PAF receptor mediated events. It is of course possible that HSD-PLA_2 is involved in the recycling of PAF via the production of lyso-PtdCho. Similarly, if oxidized phospholipids are the endogenous substrates for HSD-PLA_2 then it may simply be a housekeeping enzyme, responsible for the recycling of lyso-PtdCho. However, the action of HSD-PLA_2 on oxidized phosphatidylcholine must produce oxidized fatty acids. The oxidized fatty acids may then go on to trigger other cellular events. Thus HSD-PLA_2 may be involved in oxidative signalling which is mediated via oxidized fatty acids. Clearly much work remains to elucidate the likelihood of any of these possible roles for HSD-PLA_2.

In summary this study reports the further characterization of a serine calcium-independent phospholipase A_2 demonstrating specificity for phosphatidylcholines with oxidized fatty acids at the *sn*2 position. Studies to elucidate the biological and physiological relevance of this enzyme in normal and disease states are underway.

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